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Tripeptidase Gene (*pepT*) of *Lactococcus lactis*: Molecular Cloning and Nucleotide Sequencing of *pepT* and Construction of a Chromosomal Deletion Mutant

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The gene encoding a tripeptidase (*pepT*) of *Lactococcus lactis* subsp. *cremoris* (formerly subsp. *lactis*) MG1363 was cloned from a genomic library in pUC19 and subsequently sequenced. The tripeptidase of *L. lactis* was shown to be homologous to PepT of *Salmonella typhimurium* with 47.4% identity in the deduced amino acid sequences. *L. lactis* PepT was enzymatically active in *Escherichia coli* and allowed growth of a peptidase-negative leucine-auxotrophic *E. coli* strain by liberation of Leu from a tripeptide. Using a two-step integration-excision system, a *pepT*-negative mutant of *L. lactis* was constructed. No differences between the growth of the mutant and that of the wild-type strain in milk or in chemically defined medium with casein as the sole source of essential amino acids were observed.

The starter bacterium *Lactococcus lactis* is widely used in cheese making to provide optimal conditions for curd formation and development of the characteristic texture and flavor. Since the concentration of free amino acids in milk is low, growth of this multiply auxotrophic organism to high cell densities in milk depends on its complex proteolytic system, which produces amino acids from casein (16, 34, 42).

Peptidases form an important component of the proteolytic system; they catalyze the breakdown of peptides which are initially released from casein by the cell envelope-associated proteinase. The degraded peptides are incorporated into the cell by a di- and tripeptide transporter or an oligopeptide transport system (47; for reviews, see references 34 and 42).

In recent years, various lactococcal peptidases have been isolated and characterized both biochemically and genetically (1, 16, 34, 42). Peptidase genes that have been cloned and sequenced to date are those encoding X-prolyl dipeptidyl aminopeptidase (*pepXP*) (23, 30), the general aminopeptidases N (*pepN*) (39, 43, 48) and C (*pepC*) (5), and endopeptidase O (*pepO*) (24). The deduced amino acid sequences of these peptidases did not reveal the presence of a leader sequence, suggesting that all of these enzymes have an intracellular location. Chromosomal knockout mutations of *pepXP* and *pepO* did not affect growth of the mutant strains in milk, indicating that these individual peptidases are not essential for growth in milk (22, 24).

Recently, a tripeptidase from *L. lactis* subsp. *cremoris* Wg2 was purified to homogeneity (3). This metalloenzyme is specific for tripeptides but does not degrade tripeptides with a proline residue in the second position. A similar or identical enzyme was subsequently purified from *L. lactis* subsp. *cremoris* AM2 by Bacon et al. (2). Both enzymes are mainly located intracellularly.

As part of an ongoing effort to characterize the components of the peptidolytic system of *L. lactis*, we have cloned and sequenced the gene of a tripeptidase. A *pepT*-negative mutant was constructed and used to assess the role of the tripeptidase for growth of *L. lactis* in milk.

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MATERIALS AND METHODS

Strains, plasmids, media, and peptidase assay. The strains and plasmids used are listed in Table 1. *Escherichia coli* strains were grown in TY medium (36) at 37°C with vigorous agitation or on TY medium solidified with 1.5% agar, containing 100 μg of ampicillin per ml and 100 μg of erythromycin per ml or 50 μg of kanamycin per ml, when indicated. *L. lactis* strains were grown in M17 medium (44) at 30°C as stand cultures or in M17 medium solidified with 1.5% agar supplemented with 0.5% glucose or lactose. Erythromycin and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) were added to 5 μg/ml and 0.008%, respectively. In order to detect the activity of *E. coli* β-galactosidase in *L. lactis*, 2% glucose was added to the medium to completely suppress the lactococcal *lac* operon (53). Growth experiments in milk were carried out in 10% reconstituted skim milk (Oxoid Ltd., London, England) steamed for 20 min on two successive days. An overnight culture in 10% reconstituted skim milk was diluted 50-fold with 10% reconstituted skim milk and incubated at 30°C. Samples were taken at 30-min intervals, the pH was measured, and the optical density at 600 nm was determined by the method of Kanasaki et al. (15): 100 μl of culture was mixed with 900 μl of 0.5 M borate (pH 8.0) containing 30 mM EDTA and measured after a 30-min incubation at room temperature.

Chemically defined medium (CDM) was prepared according to Rogosa et al. (35) with the improvements of Poolman and Konings (33) and contained either a standard mixture of amino acids or 0.5% casein. Cells from a preculture in M17 medium supplemented with 0.5% glucose were washed with demineralized water, inoculated to 1% into CDM with amino acids, and grown overnight. Cells from this culture were washed with demineralized water once and inoculated to 2% into CDM with casein. The cell density was measured every hour at 600 nm according to the method described above, except that the culture was diluted only fivefold.

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant phenotype or genotype	Source or reference
L. lactis subsp. *cremoris*		
Wg2	Wild-type strain	31
NCDO 712	Wild-type strain	8
MG1363	Plasmid-free derivative of NCDO 712	8
MG1363::pINTRP-II	Derivative of MG1363 with integrated pINTRP plasmid; *pepT* Em^r β-Gal$^+$	This work
MG1363::pINTRP-II(pLP712)	Transconjugant of MG1363::pINTRP-II; *pepT* Prt^+ Lac^+ Em^r β-Gal$^+$	This work
MG1363 *pepT*(pLP712)	Segregant of MG1363::pINTRP-II(pLP712); *pepT* Prt^+ Lac^+	This work
E. coli		
NM522	*supE thi* Δ(*lac-proAB*) Δ*hsd5* (r^- m^-) (F′ *proAB lacI*^{q}*ZΔM15*)	10
CM89	*leu*-9 Δ(*pro-lac*) *met thyA pepN102 pepA11 pepB1 pepQ10*	26
pUC18/pUC19	Ap^r	55
pUK21	Km^r	54
pBluescript SK+	Ap^+	Stratagene (La Jolla, Calif.)
pORI280	Em^r β-Gal$^+$ *ori*$^+$ of pWV01; integration vector which replicates in strains containing RepA	18
pTRP6	Ap^r; 6-kb insert with entire *pepT* from MG1363	This work
pTRP96	Km^r; 3.2-kb *Mlu*I-*Xba*I fragment of pTRP6 cloned in pUK21	This work
pUK1121	Km^r; *pepT* deletion variant cloned in pUK21	This work
pINTRP	Em^r; vector for gene disruption mutation in the tripeptidase gene	This work

M9 medium (27) supplemented with 15 μM thiamine, 0.4 mM thymine, 0.4 mM methionine, 0.4 mM proline, and 0.2 mM tripeptide Leu-Gly-Gly or dipeptide Leu-Gly was used to investigate the activity of the lactococcal *pepT* gene in *E. coli* CM89 and its ability to relieve the growth defect of this strain.

Peptidase activity in toluenated cells of *E. coli* was determined by the Cd-ninhydrin method as described by Doi et al. (7) and modified according to van Boven and Konings (49): 10 μl of overnight culture was mixed with 1 μl of toluene, incubated for 20 min at room temperature, and used to detect enzyme activity.

Molecular cloning, screening, and DNA sequencing. A bank of the lactococcal chromosome was constructed in plasmid pUC19. Chromosomal DNA of *L. lactis* subsp. *cremoris* MG1363 was partially digested with *Sau*3A, partially filled in with dGTP and dATP, ligated to pUC19, cut with *Sal*I, and partially filled in with dTTP and dCTP (4). The ligation mixture was used to transform *E. coli* NM522. One thousand white colonies harboring inserts from 4 to 10 kb were grown overnight in microtiter plates. The cultures were transferred to Gene-Screen Plus filters (Du Pont Co., NEN Research Products, Boston, Mass.) placed on agar plates, incubated overnight, and used to prepare colony blots (37).

Molecular cloning techniques were performed essentially as described by Sambrook et al. (37). Plasmid DNA and chromosomal DNA from *L. lactis* were isolated by the methods of Leenhouts et al. (19, 20). *E. coli* and *L. lactis* were transformed by electroporation as described by Zabarovsky and Winberg (56) and Holo and Nes (12), respectively. Conjugation in *L. lactis* was carried out by following the protocol of Gasson and Davies (9).

The plasmid pINTRP was constructed as follows: in several steps, the *Hpa*I(a)-*Eco*RI(b) and the *Hpa*I(b)-*Xba*I fragments of pTRP96 (see Fig. 2) were cloned into plasmid pUK21 (54), resulting in the plasmid pUK1121. This procedure resulted in the replacement of the 48-bp *Eco*RI(b)-*Hpa*I(b) fragment of *pepT* (see Fig. 2 open triangle) by a 19-bp fragment from the multiple cloning site of pUC18 containing the restriction sites for *Sac*I and *Asp*718 (see Fig. 5). The deletion leads to a frameshift resulting in a truncated PepT of 233 amino acids. From pUK1121, a *Bgl*II-*Not*I fragment containing the deletion derivative of *pepT* was cloned into the integration vector pORI280 (18), cut with the same enzymes, thereby generating the plasmid pINTRP.

To determine the nucleotide sequence of the tripeptidase gene region of the lactococcal chromosome, we used a combined strategy of sequencing random subclones of the 3.2-kb *Mlu*I-*Xba*I fragment of pTRP96 and synthetic primers on pTRP96. The random subclones were generated from the purified 3.2-kb *Mlu*I-*Xba*I fragment by using the restriction enzymes *Sau*3A, *Alu*I, *Taq*I, *Rsa*I, and *Dra*I. The fragments were cloned into pUC18 and pBluescript SK+. PCR with the standard and reversed pUC primers was used to identify clones of different sizes, which were subsequently sequenced.

Sequencing reactions were carried out by the dideoxy-chain-termination method (38) with the T7 sequencing kit (Pharmacia LKB Biotechnology AB, Uppsala, Sweden) and double-stranded plasmid templates according to the manufacturer's description. Primers and oligonucleotides were synthesized with a 381A DNA synthesizer (Applied Biosystems, Inc., Foster City, Calif.). The subsequences obtained were aligned and analyzed with the PC/Gene sequence analysis program (IntelliGenetics, Inc., Geneva, Switzerland). Protein homology searches were carried out in the PIR data base (release 37) available in the ATLAS retrieval system (National Biomedical Research Foundation, Washington, D.C.) with the FASTA program (32). Sequence alignment of two proteins was carried out with the PALIGN program of PC/Gene by using the unitary matrix with standard settings. In multiple protein sequence alignment, the CLUSTAL program was used with a gap penalty of 35 and an open gap cost of 15.

Southern transfer, DNA hybridization, and PCR. After agarose gel electrophoresis, DNA was transferred to Gene-Screen Plus filters by using the protocol of Southern, as modified by Chomczynski and Quasba (6). From the N-terminal amino acid sequence of the tripeptidase (40), the following 53-mer oligonucleotide probe (OV1) was synthesized: ATG AAA TAT GAA AAA TTA TTA CCA CGT TTT TTA GAA TAT GTT AAA GTT AAT AC. The third base was chosen according to the highest score in codon usage in *L. lactis* (51).

The probe was labelled with the DIG oligonucleotide 3′-end

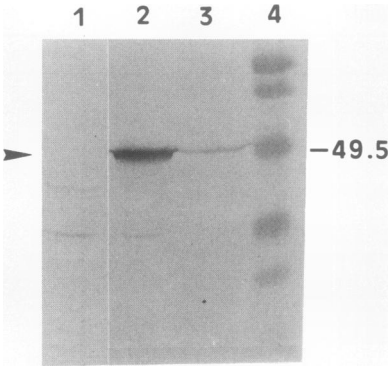

FIG. 1. Western blot analysis of cell extracts of *E. coli*(pUC19) (lane 1), *E. coli*(pTRP6) (lane 2), and *L. lactis* MG1363 (lane 3). Lane 4, standard molecular weight marker. The arrowhead indicates the proteins reacting with the tripeptidase-specific antibodies raised against the purified tripeptidase from *L. lactis* subsp. *cremoris* Wg2. Molecular size is given in kilodaltons.

labelling kit (Boehringer GmbH, Mannheim, Germany) and used for hybridization according to the manufacturer's instructions. To prevent unspecific binding of the probe to the Gene-Screen Plus filters, 1% sodium dodecyl sulfate (SDS) was included in all buffers. Hybridization of the DIG-labelled probes was detected with the DIG luminescent detection kit (Boehringer GmbH).

For hybridization with DNA fragments, the ECL labelling and detection system was used by following the directions of the manufacturer (Amersham International, Amersham, United Kingdom).

PCR was carried out with SUPER *Taq* DNA polymerase according to the instructions of the manufacturer (HT Biotechnology Ltd., Cambridge, England). Chromosomal DNA of *L. lactis* was prepared according to the method of Leenhouts et al. (20) with the following modification. After the first phenol extraction, 50 μl of the water phase was precipitated with an equal volume of isopropanol, washed once with 70% ethanol, dried, and dissolved in 50 μl of 10 mM Tris–1 mM EDTA (pH 8.0). One microliter of this preparation was used for PCR. The following primers were used: primer 1, GCT GTA CCA GGG TGA ACG, and primer 2, GTT CCT AAA GAG GTT GCA GG.

SDS-PAGE, Western blotting (immunoblotting), and immunodetection. Cell extracts of overnight cultures of *E. coli* and *L. lactis* were prepared as described by van de Guchte et al. (50). SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to Laemmli (17) with the Protean II minigel system (Bio-Rad Laboratories, Richmond, Calif.). The low-range molecular weight marker was obtained from Bio-Rad.

Proteins separated by SDS-PAGE were transferred to a BA85 nitrocellulose membrane (Schleicher & Schüll, Dassel, Germany) as described by Towbin et al. (46). Tripeptidase antigen was detected with polyclonal tripeptidase antibodies (41), diluted 1:4,000, and alkaline phosphatase-conjugated goat anti-rabbit antibodies (Promega Corporation, Madison, Wis.) according to the manufacturer's instructions.

Nucleotide sequence accession number. The *pepT* sequence has been assigned the accession number L27596.

RESULTS

Cloning and sequencing of a tripeptidase gene of *L. lactis*. On the basis of N-terminal amino acid sequencing (40) of a tripeptidase purified to homogeneity from *L. lactis* subsp. *cremoris* Wg2 (3), we designed an oligonucleotide probe to detect and isolate the corresponding DNA sequence. A 1-kb fragment of DNA was found to encode the N-terminal portion of the peptidase (data not shown). This fragment was then used as a probe to clone the entire peptidase gene, which in this case was from a library of chromosomal DNA of *L. lactis* subsp. *cremoris* MG1363. Six clones reacted with the DNA probe and were further analyzed by SDS-PAGE and Western blotting. In the cell extract of one of the clones [*E. coli* (pTRP6)], a protein of the same size as the lactococcal tripeptidase reacted specifically with the antitripeptidase antibodies (Fig. 1). From these results, we concluded that we had cloned the complete gene of a tripeptidase (PepT) of *L. lactis*.

The peptidase-encoding DNA was localized to the right-hand portion of the 6-kb insert in pTRP6 by Southern hybridization (Fig. 2). The 3.2-kb *Mlu*I-*Xba*I fragment of pTRP6 was subcloned into pUK21, and the nucleotide sequence of this fragment was determined by random sequencing and by using synthetic primers. Computer analysis of the nucleotide sequence showed the presence of two partial open reading frames (ORF1 and ORF4) and two complete ORFs (ORF2 and ORF3) (Fig. 2). Figure 3 presents the nucleotide sequence of ORF3, adjacent sequences, and the deduced amino acid sequence. ORF3 consists of 1,239 bp encoding a putative protein of 413 amino acids with a molecular weight of 45,945.

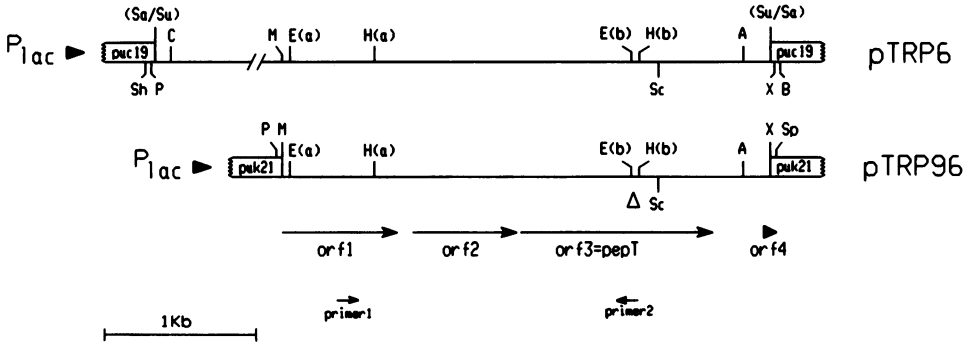

FIG. 2. Restriction enzyme map of the plasmids pTRP6 and pTRP96, showing the location of the identified ORFs. A, *Acc*I; B, *Bam*HI; C, *Cla*I; E, *Eco*RI; H, *Hpa*I; M, *Mlu*I; P, *Pst*I; Sc, *Sac*I; Sa, *Sal*I; Su, *Sau*3A; Sh, *Sph*I; Sp, *Spe*I; X, *Xba*I; P_{lac}, *lac* promoter and its direction of transcription on the plasmids pUC19 and pUK21; Δ, deletion used to construct the chromosomal *pepT* mutation. The small arrows indicate the PCR primers 1 and 2, used to identify the different types of integration events during the construction of the chromosomal deletion in *pepT*.

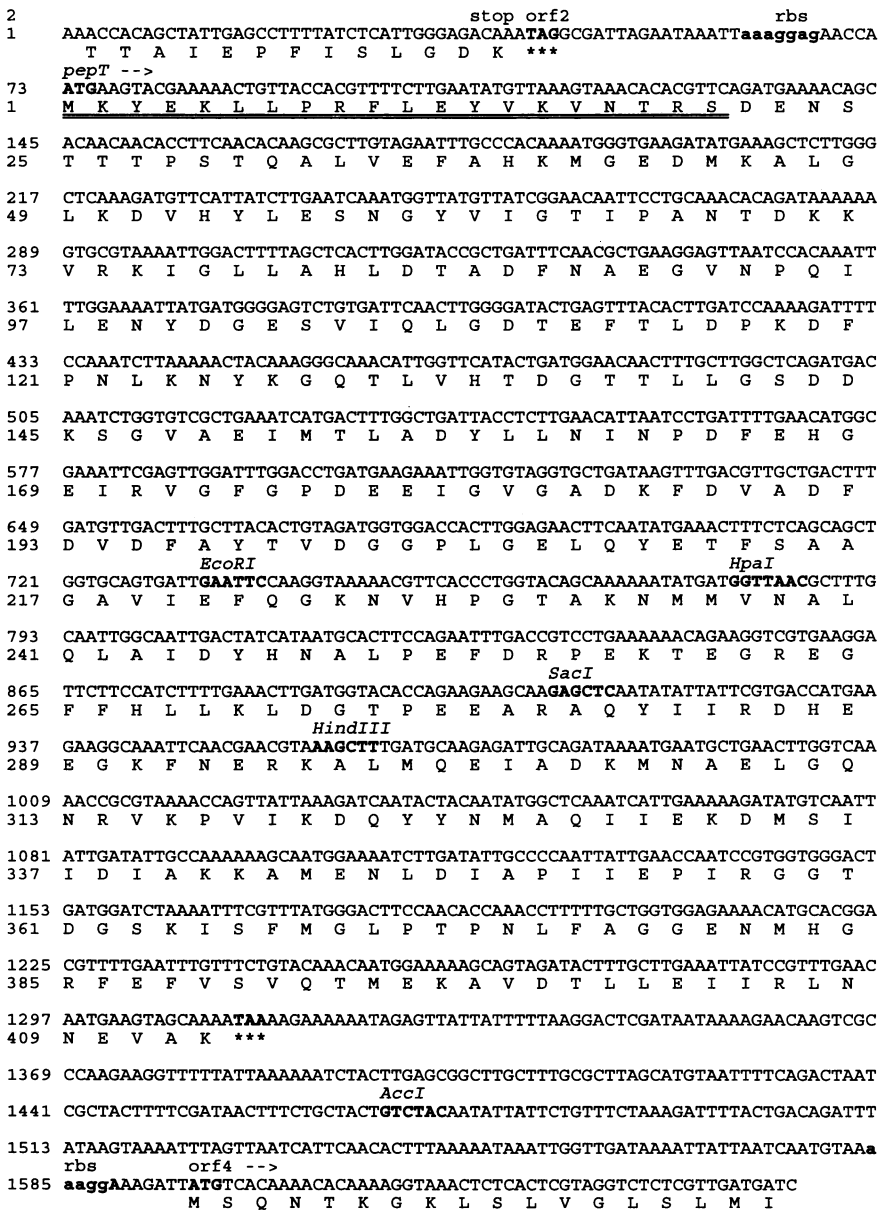

FIG. 3. Nucleotide sequence and deduced amino acid sequence of *pepT* of *L. lactis* MG1363 and part of the preceding and following ORFs. The doubly underlined amino acid sequence is identical to the N-terminal amino acid sequence determined from the purified tripeptidase. ***, stop codon; rbs, ribosome binding site (nucleotides shown in boldfaced lowercase). A number of relevant restriction enzyme sites are indicated in boldface.

Upstream of ORF3, a consensus ribosome binding site (21) with a ΔG of -14 kcal/mol (ca. -58.6 kJ/mol) (45) was present. The deduced N-terminal amino acid sequence was identical to the N-terminal amino acid sequence of the purified tripeptidase (Fig. 3). From these data, we conclude that ORF3 represents the *pepT* gene of *L. lactis*.

PepT of *L. lactis* and PepT of *Salmonella typhimurium* have extensive similarities. In order to identify similar proteins, the PIR data base (release 37) was screened with the deduced PepT amino acid sequence (Fig. 3). Extensive amino acid sequence similarity with PepT of *S. typhimurium* was found (25). The two enzymes are almost identical in size, 413 amino acids for *L. lactis* PepT and 409 amino acids for *S. typhimurium* PepT, and share 47.4% identical and 11.7% similar amino acids (Fig. 4), supporting our conclusion that we have cloned the gene of a tripeptidase.

Miller et al. (25) identified a region of amino acid similarity between PepT of *S. typhimurium* and Iap (responsible for isozyme conversion of alkaline phosphatase) of *E. coli* (14), Cpg2 (carboxypeptidase G2) of *Pseudomonas* spp. (29), and PepD (peptidase D) of *E. coli* (11). These authors speculate that this region may constitute the metal-binding site of the enzyme. Manual alignment of the corresponding region (Thr-138 to Ala-184) of the lactococcal PepT showed that 66% of the amino acids were identical to the corresponding amino acid of at least one of the four other enzymes (data not shown). In individual alignments of the PepT of *L. lactis* with Iap, Cpg2, and PepD, only Cpg2 showed extended additional regions of

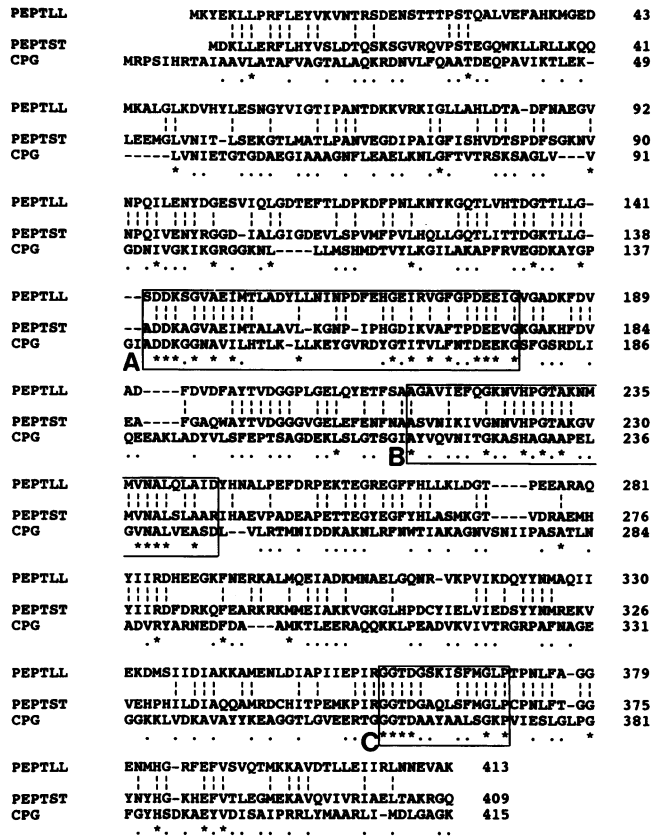

FIG. 4. Alignment of the amino acid sequences of the tripeptidases of *L. lactis* (PEPTLL) and *S. typhimurium* (PEPTST) and of Cpg2 (CPG) of *Pseudomonas* spp. Identical and similar amino acids of all three proteins are indicated by stars and dots, respectively. Regions of extensive similarity (A, B, and C) are boxed. ¦ , identical amino acids of the tripeptidases of *L. lactis* and *S. typhimurium*.

identical amino acids. In Fig. 4, the amino acid sequences of the PepT of *S. typhimurium*, that of *L. lactis*, and Cpg2 of *Pseudomonas* spp. are aligned. Apart from the region of similarity described above (region A), two other stretches of high similarity are present: region B (Ala-216 to Asp-245) (33% identical) and region C (Gly-358 to Pro-371) (43% identical) (numbering of *L. lactis* PepT), suggesting that these regions are also of structural and/or functional significance.

PepT is active in *E. coli*. To examine whether PepT is produced in *E. coli* as an active enzyme, pTRP6 was introduced into the multiple-peptidase-negative *E. coli* CM89 (26). Enzyme activity in toluene-treated cells of *E. coli*(pTRP6) was measured by the Cd-ninhydrin method, and high activity was found with the tripeptide Leu-Gly-Gly. The dipeptide Leu-Gly was not hydrolyzed (data not shown). Moreover, CM89 (pTRP6) formed colonies on minimal medium containing Leu-Gly-Gly as the sole source of leucine, for which the strain is auxotrophic. CM89 cells transformed with vector DNA were unable to grow on this medium. From these results, we conclude that PepT is active in *E. coli* and can compensate for the growth defect caused by mutations in several peptidase genes and the leucine biosynthetic operon.

Construction and analysis of A *pepT*-negative mutant. To investigate whether PepT is essential for the growth of *L. lactis* in milk, a chromosomal deletion in the *pepT* gene was constructed. The integration vector pINTRP (Fig. 5) is composed of an erythromycin resistance gene, the *E. coli* β-galactosidase gene expressed from the lactococcal promoter P32 (52), a replication origin, and a version of *pepT* in which the internal 48-bp *Eco*RI-*Hpa*I fragment has been deleted. The chromosomal mutation was constructed in a two-step approach. After transformation of MG1363 with pINTRP, erythromycin-resistant colonies which stained blue on X-Gal-containing agar plates were obtained after Campbell integration of the vector via either portion, I or II, of the insert carrying the deleted gene (Fig. 5).

By using primers 1 and 2, as indicated in Fig. 2 and 5, a fragment of 2.2 kb should be amplified if integration occurred through recombination in portion I, whereas integration through recombination in portion II should not lead to a PCR product if a short elongation time is chosen. Approximately half of the tested colonies did not yield a PCR product. As expected, these colonies did not produce PepT antigen, as evidenced by Western blot analysis (results not shown). One of the integrants obtained via recombination in portion II (MG1363::pINTRP-II) was used for further experiments.

Because of the duplication of the insert DNA in the chromosome, integrants obtained after Campbell-type recombination occasionally produce progeny in which a single copy of the insert is retained (18). In the case of MG1363::pINTRP-II, if this recombination event occurred between the duplicated regions designated A, the mutated copy of the *pepT* gene would be retained. Conversely, if the second recombination occurred in region B, the wild-type copy of the *pepT* gene would remain (Fig. 5).

To be able to investigate the growth of the ultimate *pepT* strain in milk, the proteinase genes *prtP* and *prtM* were introduced into the intermediary Em^r strain MG1363::pINTRP-II by conjugation of plasmid pLP712 (8). The resulting strain was named MG1363::pINTRP-II(pLP712).

To allow excision of pINTRP, the strain MG1363::pINTRP-II(pLP712) was grown for approximately 30 generations in the absence of erythromycin, and white erythromycin-sensitive colonies were selected. Cell extracts were prepared from the isolated clones and examined for the presence or absence of PepT by means of Western blotting. Several PepT-deficient clones were detected, and their chromosomal DNAs were isolated, cut with *Asp*718 and *Cla*I, and hybridized with labelled pTRP96. Since the deletion of 48 bp in *pepT* resulted in a new *Asp*718 site (see Materials and Methods), the chromosomes harboring the mutated *pepT* should generate two *Asp*718-*Cla*I fragments from the 9.5-kb *Cla*I fragment present in strain MG1363. Figure 6 shows that in the mutant strain MG1363 (*pepT* pLP712) two new fragments of 4 and 5.5 kb were present, demonstrating that it carried the expected chromosomal deletion.

The effect of the tripeptidase deficiency of the mutant strain on its capacity to grow in CDM with casein as the sole source of essential amino acids was examined. The results presented in Fig. 7 show that the mutant and the wild-type strain do not differ with respect to growth rates and final cell densities. In addition, no differences in acidification rates in milk were observed (data not shown). These results indicate that PepT is not essential for *L. lactis* to utilize casein or to grow in milk.

DISCUSSION

We have cloned and sequenced the gene of a tripeptidase of *L. lactis*. This conclusion is based on the following observations: (i) antibodies raised against the purified tripeptidase from *L. lactis* subsp. *cremoris* Wg2 reacted with a protein of approximately 50 kDa encoded by the cloned lactococcal

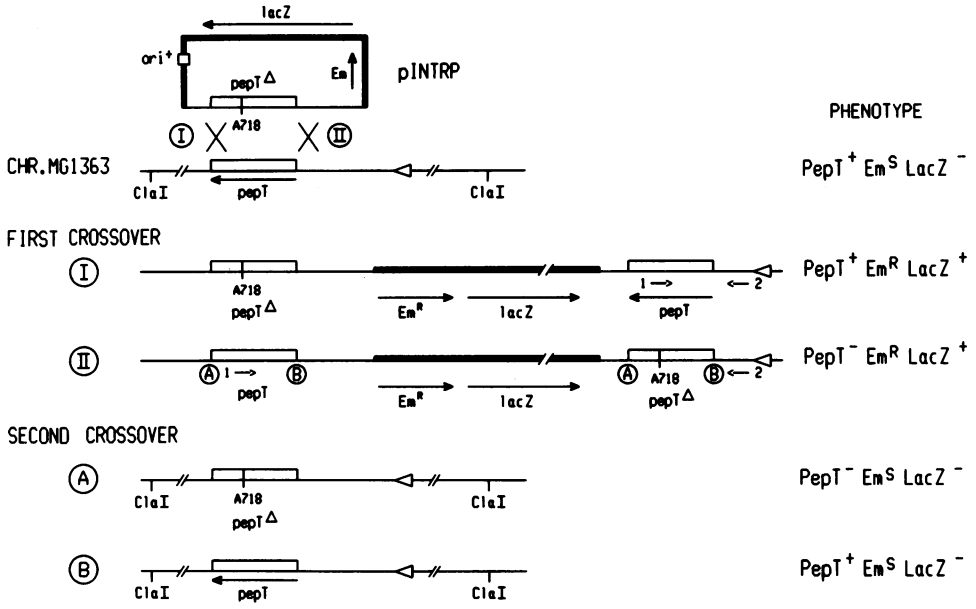

FIG. 5. Schematic representation of the construction of the chromosomal *pepT*-deletion mutation in *L. lactis* by using the plasmid pINTRP. Black bar, vector DNA; open box, *pepT*; A718, *Asp*718 site created during the construction of the *pepT* deletion derivative; pepT△, deletion derivative of *pepT*; Em, erythromycin resistance gene; lacZ, β-galactosidase gene of *E. coli* expressed under control of the lactococcal promoter P32; ori+, origin of replication of the lactococcal plasmid pWV01; I and II, possible sites for the first crossover and their products I and II; open arrow, chromosomal promoter of *pepT*; 1--> and <--2, primers used to distinguish between the two Campbell-type integrations; A and B, possible regions for a second crossover and their products A and B. Relevant phenotypes of the different constructs are shown on the right. In crossover II, *pepT* is not expressed because it is separated from its promoter and because there is no promoter on the integration plasmid in the same orientation as *pepT*.

chromosomal fragment, (ii) the N-terminal amino acid sequence deduced from *pepT* was identical to that of the purified tripeptidase, (iii) the multiple-peptidase-negative *E. coli* CM89 carrying *pepT* on a plasmid produced high tripeptidase activity, and (iv) *pepT* allowed growth of this Leu-auxotrophic *E. coli* strain by liberation of Leu from the tripeptide Leu-Gly-Gly.

As the deduced N-terminal amino acid sequence of PepT appears to be identical to that of the purified protein and no obvious membrane-spanning domains are present, PepT is most probably located in the cytoplasm of *L. lactis*. This is in agreement with the immunological data of Tan et al. (41).

In the nucleotide sequences upstream of *pepT*, no putative consensus promoter sequences could be detected. The obser-

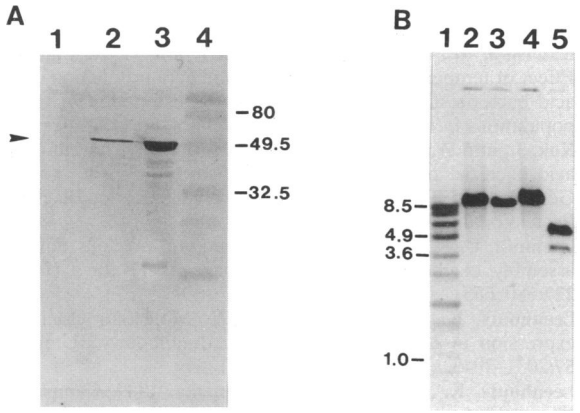

FIG. 6. Western blot (A) and Southern blot (B) analyses of the mutant *L. lactis* MG1363 *pepT*. (A) Lane 1, MG1363 *pepT*; lane 2, MG1363; lane 3, *E. coli*(pTRP6); lane 4, standard molecular weight marker. The arrowhead indicates PepT. Molecular sizes are given in kilodaltons. (B) Southern blot of digested chromosomal DNA. Lane 1, molecular weight marker (phage SppI DNA digested with *Eco*RI); lane 2, MG1363 cut by *Cla*I; lane 3, MG1363 cut by *Cla*I and *Asp*718; lane 4, MG1363 *pepT* cut by *Cla*I; lane 5, MG1363 *pepT* cut by *Cla*I and *Asp*718. Molecular sizes are given in kilobases. pTRP96 was used as probe.

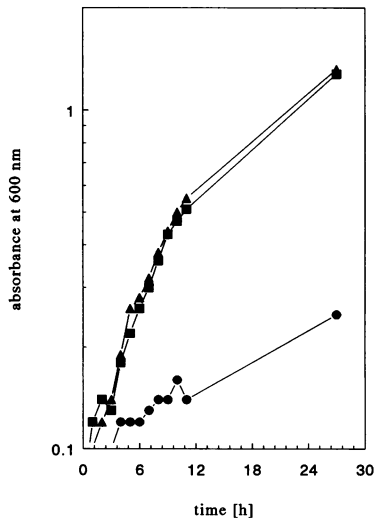

FIG. 7. Effect of a *pepT*-negative mutation on growth in CDM with casein as the sole source for essential amino acids. ▲, MG1363 (pLP712) (wild type); ●, MG1363 (proteinase-negative strain as negative control); ■, MG1363 *pepT*(pLP712).

vation that the *pepT* gene was not functionally active in the Campbell integration (II) depicted in Fig. 5 indicates that the promoter governing the transcription of *pepT* is located neither between ORF3 and ORF2 nor between ORF1 and ORF2 and should be present upstream of ORF1. Therefore, expression of *pepT* on pTRP6 is initiated in the fragment upstream of the sequenced part or at the *lac* promoter. Downstream of *pepT*, no terminator structure was present. Instead, 281 bp downstream of *pepT*, the beginning of another ORF (ORF4) was identified. These results suggest that *pepT* is part of an operon. None of the deduced amino acid sequences of the other ORFs showed similarities to other proteins in the PIR data base. Additional studies are required to further characterize the putative transcriptional unit which includes *pepT*.

PepT of *L. lactis* is very similar to the tripeptidase PepT of *S. typhimurium* (25) and the partially sequenced PepT of *E. coli* (20a), indicating a common evolutionary origin. Miller et al. (25) identified a region of homology of the *S. typhimurium* PepT with Iap (*E. coli*) (14), Cpg2 (*Pseudomonas* spp.) (29), and PepD (*E. coli*) (11) and speculated that this site is involved in metal binding rather than in specificity because the enzymes act on very different substrates. This same region is also present in the lactococcal tripeptidase (Fig. 4, region A. S-142 to G-181). In our study, two additional sites of sequence similarity between both the lactococcal PepT and the salmonella PepT and the pseudomonas Cpg2 were identified: region B (Ala-216 to Asp-245) (33% identical) and region C (Gly-358 to Pro-371) (43% identical) (*L. lactis* PepT numbering). This points to additional catalytically or structurally important domains in the enzyme.

Milk is a complex medium which contains proteins, such as caseins, peptides of various sizes, and a limited amount of free amino acids, which are utilized by *L. lactis* to complement its inability to synthesize a number of amino acids (28). The proteolytic system of *L. lactis* consists of a cell envelope-associated proteinase, peptidases with various specificities and transport systems for amino acids, di- and tripeptides, and oligopeptides, that make these amino acid sources available to the cell. To understand the role of the tripeptidase in this intricately interwoven system, a mutant strain lacking PepT was constructed. During growth both in milk and in casein-CDM, no differences between the mutant and the wild-type strains were observed, indicating that the tripeptidase is not critical for the liberation of essential amino acids from casein. Similarly, *pepXP* and *pepO* mutants were not impaired in growth in milk (22, 24). Apparently, the peptidase activity lacking in these strains can be replaced by other peptidases present in the cell. Intracellular compensation of peptidase mutations was also observed with *E. coli*, in which only combinations of mutations in several peptidase genes lead to detectable growth defects (26).

So far, the genes of six lactococcal peptidases have been cloned and sequenced and, thus, are available to construct strains with one or more mutated peptidase genes: *pepA*, *pepC*, *pepN*, *pepO*, *pepT*, and *pepXP* (5, 13, 23, 24, 48). As this can be done in a food-grade manner, the influence of individual peptidases and their combinations on cheese ripening, including testing by a taste panel, becomes feasible.

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